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Abstract

Autosomal-recessive immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome is mainly characterized by recurrent, often fatal, respiratory and gastrointestinal infections. About 50% of patients carry mutations in the DNA methyltransferase 3B gene (DNMT3B) (ICF1). The remaining patients carry unknown genetic defects (ICF2) but share with ICF1 patients the same immunological and epigenetic features, including hypomethylation of juxtacentromeric repeat sequences. We performed homozygosity mapping in five unrelated ICF2 patients with consanguineous parents and then performed whole-exome sequencing in one of these patients and Sanger sequencing in all to identify mutations in the zinc-finger- and BTB (bric-a-bric, tramtrack, broad complex)-domain-containing 24 (ZBTB24) gene in four consanguineously descended ICF2 patients. Additionally, we found ZBTB24 mutations in an affected sibling pair and in one patient for whom it was not known whether his parents were consang...

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Mutations in *ZBTB24* Are Associated with Immunodeficiency, Centromeric Instability, and Facial Anomalies Syndrome Type 2

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Autosomal-recessive immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome is mainly characterized by recurrent, often fatal, respiratory and gastrointestinal infections. About 50% of patients carry mutations in the DNA methyltransferase 3B gene (*DNMT3B*) (ICF1). The remaining patients carry unknown genetic defects (ICF2) but share with ICF1 patients the same immunological and epigenetic features, including hypomethylation of juxtacentromeric repeat sequences. We performed homozygosity mapping in five unrelated ICF2 patients with consanguineous parents and then performed whole-exome sequencing in one of these patients and Sanger sequencing in all to identify mutations in the zinc-finger- and BTB (bric-a-bric, tramtrack, broad complex)-domain-containing 24 (*ZBTB24*) gene in four consanguineously descended ICF2 patients. Additionally, we found *ZBTB24* mutations in an affected sibling pair and in one patient for whom it was not known whether his parents were consanguineous. *ZBTB24* belongs to a large family of transcriptional repressors that include members, such as BCL6 and PATZ1, with prominent regulatory roles in hematopoietic development and malignancy. These data thus indicate that *ZBTB24* is involved in DNA methylation of juxtacentromeric DNA and in B cell development and/or B and T cell interactions. Because *ZBTB24* is a putative DNA-binding protein highly expressed in the lymphoid lineage, we predict that by studying the molecular function of *ZBTB24*, we will improve our understanding of the molecular pathophysiology of ICF syndrome and of lymphocyte biology in general.

Patients with autosomal-recessive immunodeficiency, centromeric instability, and facial anomalies (ICF [MIM 242860]) syndrome suffer from recurrent and often fatal respiratory and gastrointestinal infections because of hypogammaglobulinemia, even in the presence of normal B cell counts. ICF syndrome also presents with facial anomalies, most notably a broad and flat nasal bridge, hypertelorism, and epicanthal folds. The molecular hallmark of this condition is the presence of chromosomal abnormalities in phytohemagglutinin (PHA)-stimulated peripheral blood lymphocytes (PBLs) of patients. These abnormalities are not restricted to hematopoietic cell lineages and include the formation of radial chromosomes involving the juxtacentromeric heterochromatin regions of chromosomes 1, 9, and 16.^{1,2}

Approximately half of ICF cases have mutations in the DNA methyltransferase 3B gene (*DNMT3B* [MIM 602900]) at chromosomal locus 20q11.2 (these cases are said to have ICF1).^{3,4} *DNMT3B* is one of the three main mammalian DNA methyltransferases that play an important but not exclusive role in de novo DNA methylation.⁵ In ICF1 patients, the enzymatic activity of *DNMT3B* is strongly, but not completely, reduced,⁶ and as a consequence, specific genomic regions of ICF patients show significant loss of DNA methylation. This hypomethylation is most strongly observed in satellite 2 (Sat2) repeats on the long arm of chromosomes 1 and 16, satellite 3 (Sat3) repeats on the long arm of chromosome 9, and in the nonsatellite repeats NBL2 on acrocentric chromosomes and D4Z4 in

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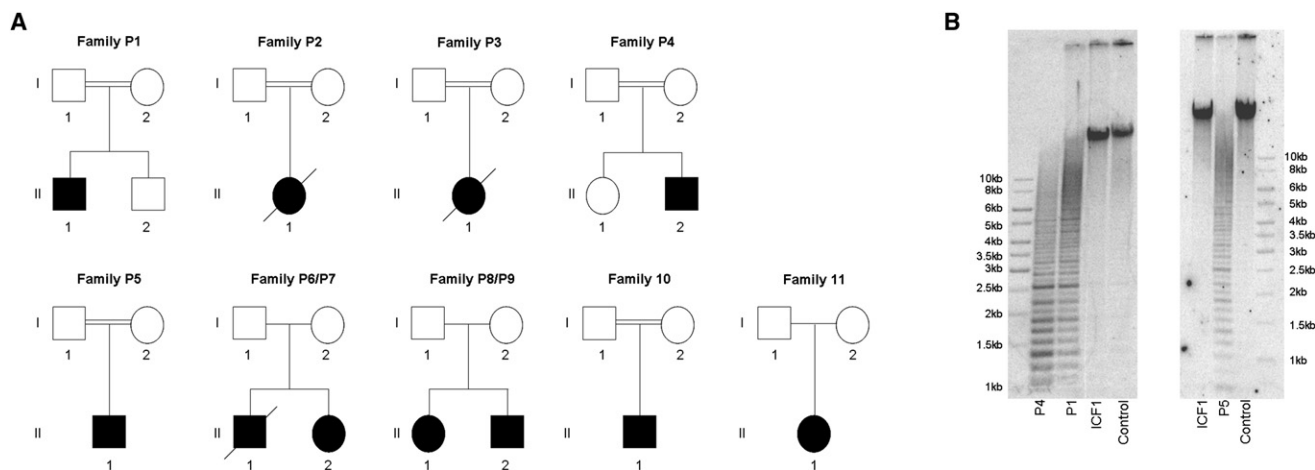


Table 1. Clinical and Genetic Data of ICF2 Patients

Case, Gender	Clinical Phenotype	Status	Origin	ZBTB24 Mutation	Number in Hagleitner et al. ²
P1, male	agammaglobulinemia, facial anomalies, motor development delay, mental retardation	4 years old	Turkish	p.Asn306IlefsX4 (c.917delA)	–
P2, female	agammaglobulinemia, facial anomalies, motor development delay, mental retardation	died at age 13	Scottish	p.Ser16X (c.47C>G)	11
P3, female	agammaglobulinemia, facial anomalies, motor development delay, mental retardation	died at age 11	Turkish	p.Arg320X (c.958C>T)	17
P4, male	agammaglobulinemia, facial anomalies, mental retardation	6 years old	Turkish	No mutation identified	–
P5, male	agammaglobulinemia, facial anomalies, mental retardation	13 years old	Turkish	p.Val168SerfsX28 (c.501dup)	–
P6, male	agammaglobulinemia, facial anomalies, mental retardation	died at age 4	German	p.Ser278X (c.833C>G) and p.Cys408Gly (c.1222T>G)	37
P7, female	hypogammaglobulinemia, facial anomalies, motor development delay, mental retardation	9 years old	German	p.Ser278X (c.833C>G) and p.Cys408Gly (c.1222T>G)	38
P8, female	agammaglobulinemia, facial anomalies, normal intelligence	11 years old	British	No mutation identified	34
P9, male	agammaglobulinemia, facial anomalies, normal intelligence	4 years old	British	No mutation identified	–
P10, male	hypogammaglobulinemia, facial anomalies, mental retardation	adult	Italian	p.Arg457X (c.1369C>T)	6
P11, female	IgM deficiency, facial anomalies, mental retardation	adult	Italian	No mutation identified	13

a British sister and brother who have no history of consanguinity. Initially, both presented with agammaglobulinemia and facial anomalies. The agammaglobulinemia in P8 and P9 was corrected by hematopoietic stem cell transplantation.^{2,16} We did not find *DNMT3B* mutations, but we observed α -satellite hypomethylation (data not shown). P10 (II.1 in family P10 in Figure 1A) is an Italian male patient who has no known history of consanguinity and who presented with hypogammaglobulinemia, facial anomalies, and mental retardation.¹⁷ We did not detect a *DNMT3B* mutation. Finally, P11 (II.1 in family P11 in Figure 1A) is an Italian female patient with a IgM deficiency, facial anomalies, mental retardation, and no known history of consanguinity.^{18,19} No mutation was found in *DNMT3B*. Because of scarcity of material, no α -satellite DNA methylation analysis was performed in P10 and P11.

To identify genes in which variation is associated with ICF, we performed homozygosity mapping²⁰ in the five unrelated ICF2 patients born to consanguineous parents (P1–P5). We used the Sentrix HumanHap-300 Genotyping BeadChips containing 317,503 loci of HapMap I and II TagSNP (Illumina). Seven hundred and fifty nanograms of DNA was processed with the Infinium II Whole-Genome Genotyping Assay (Illumina). In short, DNA was amplified, fragmented, precipitated with ethanol, and resuspended. Next, the DNA was applied to the BeadChip and incubated overnight; enzymatic base extension, fluorescent staining

of the beads, and detection of fluorescent intensities by the BeadArray Reader (Illumina) followed. Finally, to detect regions of homozygosity, we assessed B allele frequencies for all SNPs by using BeadStudio version 3.2 (Illumina). Large regions of homozygosity were identified in all patients and confirmed that the patients were each of consanguineous descent. However, no region of homozygosity was shared by all patients, suggesting that ICF2 itself is also heterogeneous.

Next, we performed exome sequencing²¹ to study all exons in the genome of one consanguineously descended ICF2 patient (P3).¹⁴ Five micrograms of DNA was fragmented to yield 200–300 bp fragments with the Bioruptor (Diagenode). End repair of DNA fragments was subsequently carried out per the manufacturer's instructions (New England Biolabs). Next, paired-end adaptor oligonucleotides (Illumina) were added to the ends of the DNA fragments, and the resulting DNA-adaptor-ligated fragments were hybridized for 72 hr against the Sequence Capture Human Exome 2.1M Array (Roche NimbleGen); washing of unbound fragments and elution of the exome-enriched pool of DNA followed. The eluted DNA fragments were amplified with adaptor-specific primers (Illumina) and Phusion DNA polymerase (Finnzymes). Finally, the exome-enriched DNA fragments were sequenced with the Genome Analyzer IIx system (Illumina). We prepared paired-end flow cells on the supplied cluster

station according to the manufacturer's instructions. The resulting reads were aligned to the reference human genome (hg 19, NCBI build 37) by Bowtie software,²² and sequence variants were identified with SAMtools software.²³ In a homozygous region that was shared between four of the five patients of consanguineous descent, we identified a homozygous sequence variant (c.958C>T; NM_014797.2) in exon 3 of the zinc-finger-and BTB (bric-a-bric, tramtrack, broad complex)-domain-containing (*ZBTB24*) gene. This sequence variant creating a premature stop codon at amino acid position 320 (p.Arg320X; NP_055612.2) was confirmed by PCR amplification of exon 3 of *ZBTB24* and subsequent Sanger sequencing (Figure 2A).

Finally, we performed mutation analysis in all *ZBTB24* coding exons by PCR amplification and Sanger sequencing in the remaining four consanguineously descended ICF2 patients (P1, P2, P4, and P5) and in the six additional ICF2 patients for whom genomic DNA was available (P6–P11). The primers used for the *ZBTB24* mutation analysis are shown in Table S1, available online. We identified homozygous *ZBTB24* sequence variants in three additional consanguineously descended patients (P1, P2, and P5) and in one patient for whom consanguineous descent had not been previously established (P10). An affected sibling pair of non-consanguineous descent was compound heterozygote for *ZBTB24* mutations (P6 and P7). We did not find any *ZBTB24* sequence variants in an affected sibling pair (P8 and P9), one patient of nonconsanguineous descent (P11), or one consanguineously descended patient (P4), confirming additional genetic heterogeneity. P4 was also excluded for the *ZBTB24* locus based on our homozygosity mapping data.

P1 is homozygous for a 1 bp deletion in exon 2 (c.917delA; NM_014797.2), resulting in a frameshift and a premature stop codon three amino acids downstream (p.Asn306IlefsX4; NP_055612.2). P2 carries a homozygous nonsense mutation in exon 2 (c.47C>G; NM_014797.2), resulting in a serine-to-stop conversion (p.Ser16X; NP_055612.2). P5 is homozygous for a 1 bp duplication in exon 2 (c.501dup; NM_014797.2), resulting in a frameshift and a premature stop codon 27 amino acids downstream (p.Val168SerfsX28; NP_055612.2). P10 carries a homozygous nonsense mutation in exon 6 (c.1369C>T; NM_014797.2), resulting in an arginine-to-stop conversion (p.Arg457X; NP_055612.2). Finally, P6 and P7 carried a serine-to-stop nonsense mutation in exon 2 (c.833C>G, p.Ser278X; NM_014797.2 and NP_055612.2) and a cysteine-to-glycine missense mutation in exon 5 (c.1222T>G, p.Cys408Gly; NM_014797.2 and NP_055612.2); the latter mutation affects a conserved cysteine in one of the eight C₂H₂ zinc fingers of the ZBTB24 protein. In conclusion, all but one of the identified mutations created premature stop codons in *ZBTB24*. This suggests that ICF2 is caused by loss-of-function mutations in *ZBTB24*. The identified *ZBTB24* mutations are summarized in Table 1; Sanger sequencing results are shown in Figure 2A, and the locations of the mutations in ZBTB24 are shown in

Figure 2B. Finally, in Table S2 the clinical and immunological characteristics that the ICF2 patients with *ZBTB24* mutations had at diagnosis are summarized and compared with those that ICF1 patients with *DNMT3B* mutations had at diagnosis, and no difference in phenotype between ICF1 and ICF2 patients is revealed.

Using quantitative reverse transcriptase PCR (qRT-PCR), we studied full-length *ZBTB24* mRNA expression in human primary cell cultures, tissues, and cell lines. In addition, we combined fluorescence-activated cell sorting (FACS) analysis and qRT-PCR to study *ZBTB24* mRNA expression in subpopulations of normal human B cells, CD4⁺ T cells, and natural killer (NK) cells more specifically. We obtained human peripheral blood from two healthy blood-bank donors after receiving informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over Ficoll-Hypaque density gradients. CD19⁺ B cells were purified by positive selection with the Dynabeads CD19 Pan B (Invitrogen) and then detached by DETACHaBEAD CD19 (Invitrogen). Purified B cells were then stained with a combination of CD19^{percp-cy5.5} (SJ25C1), CD27^{phycoerythrin} (L128), IgM^{allophycocyanin} (G20-127), and IgD^{fluorescein isothiocyanate} (IA6-2) antibodies. CD4⁺ T cells were purified by positive selection with the Dynabeads FlowComp Human CD4 T cell isolation kit (Invitrogen). Purified T cells were then incubated with CD4^{pacific blue} (RPA-T4), CD45RA^{fluorescein isothiocyanate} (L48), and CCR7^{alexa fluor 647} (3D12) antibodies. All antibodies were obtained from BD Biosciences. The following cell fractions were sorted with a FACS Aria cell sorter (Becton Dickinson) with greater than 98% purity: total CD19⁺, naive CD19⁺CD27⁺IgD⁺, unswitched memory CD19⁺CD27⁺IgD⁺IgM⁺ and switched memory CD19⁺CD27⁺IgD⁺IgM⁺ B cells, total CD4⁺, naive CD4⁺CD45RA⁺CCR7⁺, and memory CD4⁺CD45RA⁺ T cells. The analysis and sort gates were restricted to the small lymphocyte gate as determined by their characteristic forward and side-scatter properties. NK cells were isolated with the MACS NK Cell Isolation Kit (Miltenyi Biotech). NK cell purity was at least 95% of isolated cells. Next, total RNA was isolated from a minimum of 100,000 cells with the NucleoSpin RNA II kit (Macherey-Nagel). The RNA sample was then used for cDNA synthesis, which was performed with random hexamers (MBI-Fermentas) and RevertAid H Minus Reverse Transcriptase (MBI-Fermentas). The final cDNA sample was used in a RT-PCR reaction containing iQ SYBR Green Supermix (Bio-Rad) and 1 μM of both forward and reverse primers. All RT-PCR reactions were performed in duplicate with the MyiQ2 Two-Color Real-Time PCR Detection System (Bio-Rad). Primer sequences are shown in Table S3. These experiments showed that *ZBTB24* is ubiquitously expressed, and the highest expression levels are in B cell subpopulations, most notably in naive B cells (Figure 3). This corroborates the role of ZBTB24 in B cell differentiation and is consistent with the observed B cell defects in ICF patients.²⁴

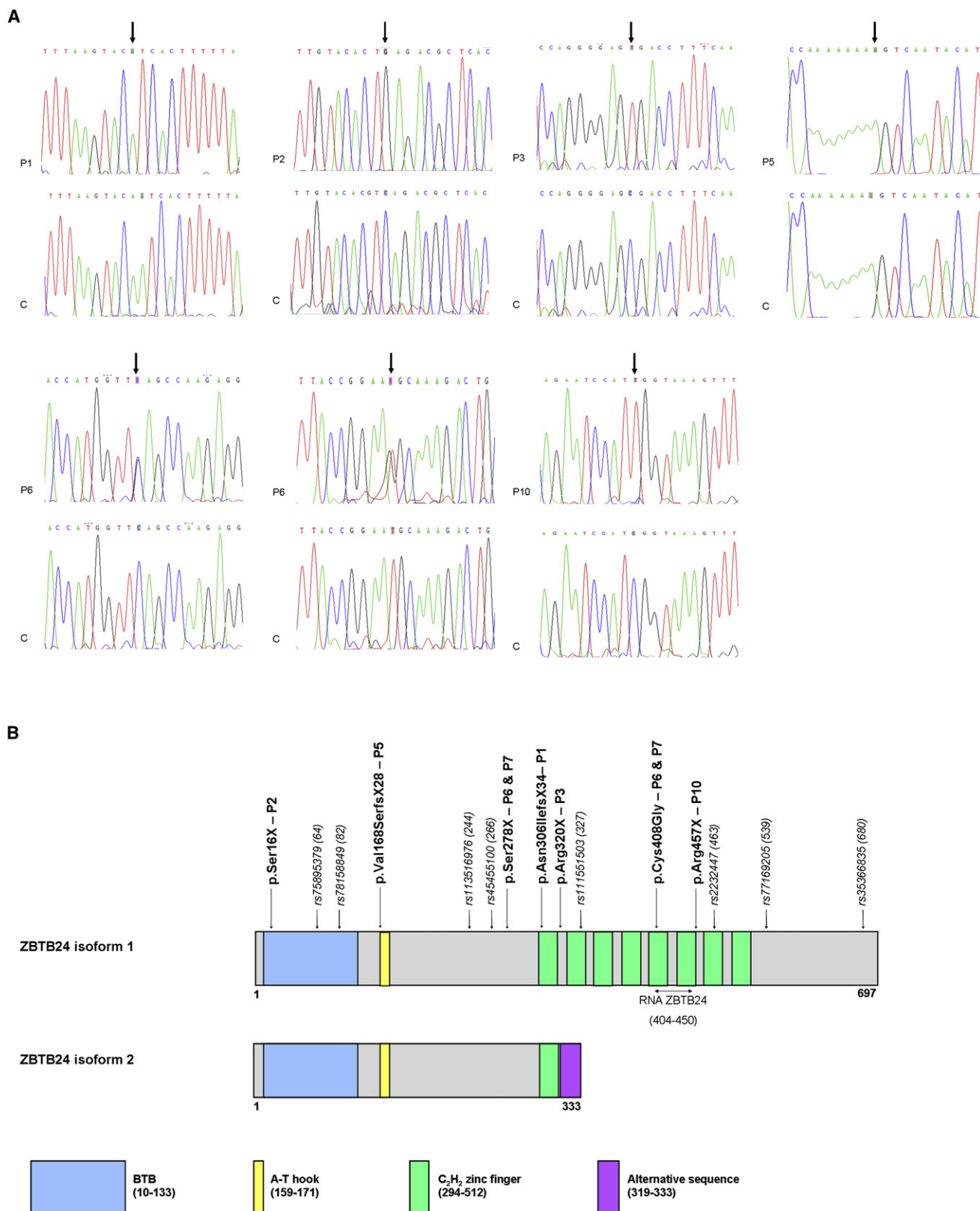


Figure 2. ZBTB24 Mutations at the DNA and Protein Level

(A) Sanger sequencing results of seven ICF2 patients with *ZBTB24* mutations and an unrelated control individual. P1 carries a homozygous 1 bp deletion resulting in a frameshift and a premature stop codon three amino acids downstream (p.Asn306IlefsX4). Both parents are heterozygous carriers for the mutation, and the healthy brother is heterozygous as well (data not shown). P2 is homozygous for a nonsense mutation resulting in a serine-to-stop conversion (p.Ser16X). P3 carries a homozygous sequence variant creating a premature stop codon (p.Arg320X). Both parents are heterozygous carriers for the mutation (data not shown). P5 carries a homozygous 1 bp

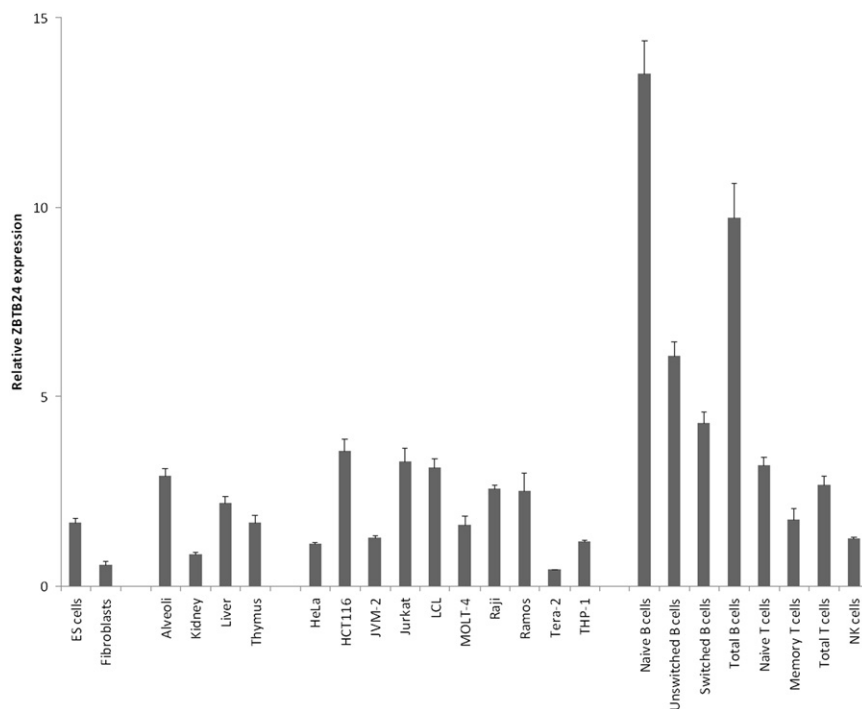


Figure 3. ZBTB24 Expression

Full-length *ZBTB24* expression levels were measured in human primary cell cultures (ES cells and fibroblasts; $n = 2$), in human tissues (alveoli, kidney, liver, and thymus; $n = 1$), in human cell lines (HeLa, HCT116, JVM-2, Jurkat, LCL, MOLT-4, Raji, Ramos, Tera-2, and THP-1; $n = 1$), in human B cells (naive B cells [CD19⁺CD27⁺IgD⁺], unswitched memory B cells [CD19⁺CD27⁺IgD⁺IgM⁺], switched memory B cells [CD19⁺CD27⁺IgD⁺IgM⁺], and total B cells [CD19⁺]; $n = 2$), in human CD4⁺ T cells (naive CD4⁺ T cells [CD4⁺CD45RA⁺CCR7⁺], memory CD4⁺ T cells [CD4⁺CD45RA⁺], and total CD4⁺ T cells [CD4⁺]; $n = 2$), and in human NK cells (CD3⁺CD56⁺; $n = 2$). Expression levels were measured in duplicate and are presented relative to the expression levels of the three house-keeping genes *GAPDH*, *GUSB*, and *ACTB*, which were also measured in duplicate. Data are presented as mean \pm standard deviation.

ZBTB24 (also known as ZNF450, BIF1, or PATZ2) contains a BTB domain, a DNA-binding A-T hook domain, and eight C₂H₂ zinc finger domains (Figure 2B). BTB domains are found near the N terminus of several zinc finger proteins. These domains mediate homomeric or heteromeric dimerization between proteins²⁵ and transcriptional repression²⁶ and have been shown to interact with histone deacetylase corepressor complexes such as NCoR (nuclear receptor corepressor), SMRT (silencing mediator of retinoic acid and thyroid hormone receptor),²⁷ and BCoR (BCL6 corepressor).²⁸ A-T hook domains are DNA-binding motifs present in many proteins, including the chromosomal high-mobility group A (HMGA) proteins that are involved in gene transcription, retroviral integration, neoplastic transformation, and cancer cell metastasis.²⁹

ZBTB24 is a member of a family of more than 40 human ZBTB proteins, several of which play prominent roles in hematologic differentiation.³⁰ A well-studied ZBTB protein is BCL6 (ZBTB27 [MIM 109565]), which was initially identified as deregulated in certain B cell lymphomas by chromosomal translocations.³¹ BCL6 seems to be required for germinal center (GC) formation,³² represses gene functioning during the terminal differentiation of B cells into

plasma cells,³³ and binds to the p53 promoter, thereby downregulating p53 expression in GC B lymphocytes and lymphomas.³⁴ Other ZBTB proteins, including BCL6B (ZBTB28 [MIM 608992]),^{35,36} PATZ1 (ZBTB19 [MIM 605165]),³⁷ ZBTB7B (MIM 607646),^{38,39} and ZBTB32 (MIM 605859),^{40,41} have been implicated in hematologic development and malignancies.⁴²

Explaining the molecular mechanisms underlying ICF1 syndrome has been a challenge. DNMT3B is a ubiquitously expressed enzyme central to mammalian biology. *Dnmt3b*-knockout mice are embryonic lethal, and ICF1 syndrome alleles should be considered hypomorphs.⁵ Two mechanisms have been proposed to link DNMT3B defects to compromised B cell immune responses. First, DNA methylation of promoter regions of genes involved in B cell differentiation might be disturbed. Indeed, a number of transcriptionally deregulated genes have been identified in EBV-transformed B cell lines of ICF patients, but only a small proportion of them have significantly altered DNA methylation promoter levels.^{43,44} Nevertheless, it is conceivable that altered chromatin structures of some master genes of B cell development contribute to ICF pathology. A second mechanism postulates that the altered

insertion resulting in a frameshift and a premature stop codon 27 amino acids downstream (p.Val168SerfsX28). P6 and P7 are compound heterozygous for a serine-to-stop nonsense mutation (p.Ser278X) and a cysteine-to-glycine missense mutation (p.Cys408Gly). P10 is homozygous for a nonsense mutation resulting in an arginine-to-stop conversion (p.Arg457X). The mother is a heterozygous carrier for the mutation (data not shown).

(B) A schematic of the two isoforms of the ZBTB24 protein. The full-length ZBTB24 protein (isoform 1) contains a BTB domain (amino acids 10–133), a DNA-binding A-T hook domain (amino acids 159–171), and eight C₂H₂ zinc finger domains of 23 amino acids each (amino acids 294–512). The shorter isoform 2 lacks seven C₂H₂ zinc finger domains, and the last 15 amino acids are an alternative sequence (an alternative stop codon is used in the intron between exon 2 and exon 3). RNA ZBTB24 denotes the location of the primers used for RT-PCR. At least eight SNPs have been found in the coding sequence of *ZBTB24*. The numbers and locations of these SNPs are indicated as well.

chromatin structure of juxtacentromeric heterochromatin and changes in the nuclear architecture lead to defects in gene expression through effects in *trans*. Indeed, several reports support this hypothesis by demonstrating altered nuclear architecture and changes in chromosome territories with consequences for gene expression.^{45,46}

Intriguingly, mice carrying ICF-like missense mutations in *Dnmt3b* recapitulate some, but not all, molecular and clinical features of ICF syndrome. These mice have hypomethylated repetitive DNA, low body weight, and cranial facial abnormalities. However, these mice do not present with a B cell defect; they have a normal spectrum of B cell precursors and mature B cell populations. Instead, these mice have reduced amounts of thymocytes and increased levels of fragmented nuclei in the thymus.⁴⁷ Although T cell defects have been rarely reported in ICF syndrome, the presence of opportunistic infections in ICF patients strongly argues for a (subtle) T cell defect in ICF syndrome.² In ICF patients, the early stages of B cell development are reported to be normal, and the defect mainly seems to arise in the peripheral terminal steps of B cell differentiation. ICF patients have immature naive B cells and impaired negative B cell selection. Although patients have strongly reduced or absent immunoglobulin levels, ICF B cells produce normal amounts of immunoglobulins upon in vitro stimulation.²⁴ These findings strongly argue that the defect in ICF syndrome is restricted to the latest stages of B cell development, possibly in combination with an impaired T and B cell interaction.

The expression of *ZBTB24* seems to be coregulated with *DNMT3B* during B cell differentiation (Figure S1). Therefore, to investigate direct effects of *DNMT3B* mutations on *ZBTB24* expression or vice versa, we studied the major known splice variants of *DNMT3B* (*DNMT3B1*, *DNMT3B2*, *DNMT3B3*, *DNMT3B4*, and *DNMT3B5*) and the presence of *ZBTB24* in ICF1, ICF2, and control RNA samples by nonquantitative RT-PCR. RNA and cDNA samples were prepared in the same way as before, but the final cDNA sample was used in a standard PCR reaction containing DreamTaq DNA polymerase (Fermentas) and 1 μ M of both forward and reverse primers (Table S3). All *DNMT3B* splice variants and the *ZBTB24* transcript could be detected in ICF1, ICF2, and control samples (Figure S2). Because our RNA sources came from different origins, both primary cells and immortalized cell lines, we refrained from quantitative analysis of *DNMT3B* and *ZBTB24* expression.

With the identification of mutations in *ZBTB24*, encoding a member of a family of transcription factors important for B and T cell differentiation, we expect that molecular studies of *ZBTB24* will soon shed more light on the pathophysiology of ICF syndrome and lymphocyte biology in general.

Supplemental Data

Supplemental Data include two figures and three tables and can be found with this article online at <http://www.cell.com/AJHG/>.

Acknowledgments

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Web Resources

The URL for data presented herein is as follows:

Online Mendelian Inheritance in Man (OMIM), <http://www.omim.org>

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